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A Novel Destabilizing Domain Based on a Small-Molecule Dependent Fluorophore

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Abstract

Tools that can directly regulate the activity of any protein-of-interest are valuable in the study of complex biological processes. Herein, we describe the development of a novel protein domain that exhibits small molecule-dependent stability and fluorescence based on the bilirubin-inducible fluorescent protein, UnaG. When genetically fused to any protein-of-interest, this fluorescent destabilizing domain (FDD) confers its instability to the entire fusion protein, facilitating the rapid degradation of the fusion. In the presence of its cognate ligand bilirubin (BR), the FDD fusion becomes stable and fluorescent. This new chemical genetic tool allows for rapid, reversible, and tunable control over the stability and fluorescence of a wide range of protein targets.

Graphical Abstract

The ability to control the abundance of a specific protein in cells represents a powerful approach to interrogating complex biological behavior. One of the most well established ways to modulate protein activity is to knockdown the corresponding gene-of-interest, either by targeting its precursor DNA or RNA molecules. While recent advances in genome editing tools have significantly improved the efficiency of perturbing specific genes,¹ such efforts can be laborious and are not readily reversible. Alternatively, RNAi allows researchers to more quickly assess the effects of gene silencing;² however, this approach is often plagued by incomplete knockdown, off-target specificity, or other nonspecific interactions.³ Because each of these approaches modulates protein activity indirectly, they ultimately suffer from long experimental delays, given that the previously transcribed and synthesized protein molecules must be degraded before effects can be observed.

To circumvent these challenges, several laboratories have developed tools to directly control protein levels using cell permeable small molecules.⁴ The majority of these strategies

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ASSOCIATED CONTENT

Supporting Information

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Experimental procedures, supporting Table S1, and supporting Figures S1–S3 (PDF)

The authors declare no competing financial interest.

directly recruit enzymes involved in the ubiquitin-proteasome system (UPS) to the protein-of-interest (POI), thereby promoting its degradation. One such system employs proteolysis targeting chimeric molecules (PROTACs), which comprise a ligand for an E3 ligase that is tethered to a small molecule possessing high affinity for a protein target. The induced proximity of the E3 and protein target is sufficient to facilitate its degradation by the proteasome. Though highly selective and potent PROTACs have been developed,^{5,6} the generality of this approach is limited by the relatively few protein targets that possess a known small molecule binding partner.

A more general approach relies on genetically tagging a POI with a protein domain that is already known to exhibit ligand-dependent stability. In one example, an auxin-mediated degradation system identified in plants was exploited to impart small-molecule control over protein levels in mammalian cells. When fused to an auxin-inducible degron, the tagged protein undergoes association with an F box protein and an SCF E3 ligase in the presence of auxin, thereby mediating its polyubiquitylation and degradation.⁷ This strategy exhibits rapid and reversible protein degradation; however, it necessitates the coexpression of the plant-specific F box protein to properly degrade the target. More recently, Lin and coworkers engineered a protein domain that comprises a degron fused to an HCV protease and its cognate recognition site.⁸ In the absence of an HCV protease inhibitor, the protease excises itself and the degron from the fusion protein, thereby releasing the untagged protein. The addition of the inhibitor prevents the protease from engaging its cleavage site; thus, the fusion protein remains tagged with a degron and is rapidly degraded. This strategy is particularly attractive for a protein target that cannot function properly when either of its termini is covalently modified. The loss of the tag unfortunately comes with a cost, given that the degradation of untagged protein that is expressed in the absence of a drug is no longer under small molecule control.

In contrast to these systems, our laboratory has previously developed a conditional protein stability system in which the folding state of a protein target is dependent on a small molecule ligand. Specifically, we engineered mutants of the human FKBP12 protein that are properly folded and metabolically stable in the presence of their high affinity ligand, Shield-1 (S1).⁹ In the absence of S1, these destabilizing domains (DDs) significantly populate an unfolded state and are rapidly degraded by the ubiquitin-proteasome system.¹⁰ Importantly, this instability is conferred to any protein tagged with a DD, resulting in the degradation of the entire fusion protein. In addition to the FKBP-based system, we have developed DDs based on *E. coli* dihydrofolate reductase (DHFR)¹¹ and the human estrogen receptor ligand binding domain (ERLBD)¹² using their respective small molecule binding partners, trimethoprim and tamoxifen. Notably, a self-cleaving version of the DDs has been engineered by taking advantage of a split ubiquitin system.¹³ The genetic fusion of any of these DDs to a protein target ensures specificity, while the small molecule component confers rapid, reversible, and dose-dependent control over protein levels in mammalian cells.

To complement our current palette of DD systems, we sought to develop a destabilizing domain based on a recently identified fluorescent protein, UnaG (Figure 1B).¹⁴ This 139-residue polypeptide was isolated from the Japanese freshwater eel and belongs to the fatty acid binding protein (FABP) family of transport proteins. Interestingly, UnaG was found to

exhibit small molecule-dependent fluorescence: when bound to its high affinity ligand, bilirubin (BR), UnaG becomes a green fluorophore whose intrinsic fluorescence surpasses that of enhanced green fluorescent protein (eGFP). However, the apo form of the small protein is not fluorogenic. Given its unique ligand-dependent spectroscopic properties, we hypothesized we could engineer an UnaG mutant whose fluorescence and stability were both under the control of BR (Figure 1A). Fusion of this fluorescent destabilizing domain (FDD) to a protein-of-interest was envisioned to deliver a protein that is both stable and fluorescent when exposed to BR, but unstable and no longer fluorescent in the absence of the ligand. Notably, this new DD would provide precise control over protein concentration while also possessing the capabilities of a fluorescent protein tag. This latter feature was anticipated to (1) simplify the quantification of the tagged protein and (2) provide a handle by which to assess its cellular localization.

To engineer UnaG mutants that display ligand-dependent stability, we implemented a cell-based screening protocol in which a red fluorescent protein (mCherry) served as a visual readout for UnaG stability. A library of UnaG mutants was generated using error-prone PCR and cloned behind mCherry, and a retroviral expression system was used to stably integrate this library into NIH 3T3 cells. We then subjected transduced cells to several rounds of fluorescence-activated cell sorting (FACS): in the first round, cells were treated with bilirubin, and those that displayed high UnaG and mCherry fluorescence were collected. This new population of cells was then cultured in the absence of a ligand, and only cells expressing low levels of the fusion protein were isolated. After a third and fourth round of sorting in the presence and absence of BR, respectively, we extracted genomic DNA from cells, amplified the DD candidates by PCR, and isolated 60 UnaG clones.

Each individual UnaG-mCherry clone was stably retransduced into 3T3 cells, and the fluorescence of these clonally pure populations was evaluated in the presence and absence of BR. Gratifyingly, we found that several UnaG mutants displayed the desired ligand-dependent behavior, as evidenced by the significant increases in both UnaG and mCherry fluorescence when cells were exposed to BR (Figure 2 and Supporting Information, Table S1). From this collection of DDs, we sought to further characterize a mutant that displayed low basal expression levels in the absence of BR while also presenting a large dynamic range. For these reasons, we chose an UnaG double mutant, clone 15 (A36V, R136G; hereafter referred to as FDD), for further studies.

Given that this candidate FDD was isolated from a library of C-terminal UnaG mutants, we first set out to determine its behavior when cloned as an N-terminal fusion. Cells stably expressing FDD-mCherry or mCherry-FDD were exposed to 10 μ M BR, and expression of the fusion proteins was evaluated in living cells by fluorescence imaging. For both the C- and N-terminal fusions, we observed significant induction of both the UnaG mutant and mCherry in the presence of bilirubin, but low basal fluorescence in its absence (Figure 3A and Supporting Information, Figure S1). Cells expressing the C-terminal FDD were then treated with various concentrations of BR, and their fluorescence was assessed using flow cytometry. Notably, both the FDD and mCherry display dose-dependent fluorescence, and mCherry levels correlate well with FDD expression (Figure 3B). This finding demonstrates

that FDD fluorescence intensity serves as a reliable readout of the relative concentration of the fusion protein.

We next sought to evaluate the kinetics of FDD degradation. Cells expressing the mCherry-FDD fusion were treated with BR for 24 h, at which point BR was removed from the culture media.¹⁵ Analysis of the cells at several time points following withdrawal of BR revealed that the fusion protein is rapidly degraded: within a few hours, fluorescence intensities drop to near basal expression levels (Figure 3C). To gain insight into the cellular machinery responsible for FDD proteolysis, 3T3 cells stably transduced with mCherry-FDD were initially treated with BR to stabilize the FDD fusion. Immediately following removal of BR, cells were cultured with or without the proteasome inhibitor MG132 for 8 h. Cells exposed to MG132 displayed a significant accumulation of mCherry-FDD even after the stabilizing ligand was removed, as evidenced by flow cytometry and Western blot analysis (Figure 3D and E). Alternatively, exposure of cells to chloroquine, an inhibitor of lysosomal degradation, under otherwise identical conditions did not impede degradation of the fusion protein (Supporting Information, Figure S2). These collective data suggest that FDD degradation is predominantly a proteasome-dependent process, which is consistent with results obtained using our previous DD systems.

Thus far, we have examined the behavior of the FDD in the context of a fluorescent reporter gene. To determine the potential generality of this novel stability system, we fused the FDD to several different protein targets, including the proteasome interacting factors zfa2a and zfa2b, the ubiquitin conjugating enzyme ube2n, and the cell cycle regulator p21. Cells transduced with each of these fusion proteins were incubated with or without stabilizing ligand for 24 h, at which point cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. We were pleased to find that BR-dependent stability is conferred to each of these fusion proteins (Figure 4A), indicating that the FDD can impart small-molecule control over a functionally diverse set of biologically relevant proteins. Moreover, tunable control over the intracellular concentration of these fusion proteins can be achieved. For example, treatment of cells stably expressing ube2n-FDD or zfa2a-FDD with varying concentrations of BR elicits dose-dependent expression of the fusion proteins (Supporting Information, Figure S3).

Rather than depend on immunoblot assays to verify the conditional expression of each protein target, we sought to capitalize on the intrinsic fluorescence of our novel DD and more readily evaluate protein levels using fluorescence microscopy. This visual analysis would offer the added advantage of providing spatial information regarding a target's cellular destination. To this end, we stabilized ube2n-FDD with BR for 24 h and examined fluorescence by live cell imaging. We observed green fluorescence that was evenly distributed throughout the cell, which is consistent with ube2n's prominent role in the ubiquitylation of a diverse array of protein targets (Figure 4B).^{16,17} On the other hand, induction of FDD-p21 results in a fusion protein that primarily resides in the nucleus, supporting its well-characterized role in mediating cell cycle progression.¹⁸ Finally, we observed robust induction of both zfa2a and zfa2b: whereas the former displays diffuse expression,¹⁹ the latter was observed to accumulate in the cytoplasm.

In summary, we have developed a novel conditional protein stability system based on the ligand-dependent fluorophore, UnaG. We engineered an UnaG mutant to be rapidly degraded in the absence of its cognate ligand, bilirubin. When fused to a protein-of-interest, this new DD confers its instability to the entire fusion; however, treatment of the fusion with BR rescues the target from degradation. Importantly, this new DD retains the fluorogenic properties of its wild-type parent, which provides an additional handle by which to characterize the FDD-tagged target. Indeed, the functionally diverse set of proteins we tagged with the FDD were all found to exhibit BR-dependent stability and fluorescence. Given the dual functionality of this new system, it may prove particularly useful in the study of proteins whose intracellular localization, abundance, and function are highly dynamic, such as the autophagy associated protein p62 (SQSTM1).²⁰ A caveat to this approach is that BR possesses cytotoxic properties at high concentrations, an effect most pronounced in the context of neural cells.²¹ However, within the realm of the parameters evaluated in the present study, no cytotoxic effects were observed. Researchers using this technology will be able to rapidly, reversibly, and tunably regulate both the abundance and fluorescence of any protein of interest using a single genetic manipulation. Further studies exploring the utility of this new class of DDs and molecular mechanisms of degradation are ongoing in our laboratory.

METHODS

Details of experimental procedures are provided in the [Supporting Information](#).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Development of the FDD. (A) Strategy for the development of a fluorescent destabilizing domain (FDD). The genetic fusion of the FDD to a protein-of-interest (POI) will deliver a fusion protein that exhibits ligand dependent stability and fluorescence. (B) Structure of wild-type UnaG and its cognate ligand, bilirubin (BR; PDB: 4I3B).

Figure 2.

BR-sensitive mutants isolated from the library screen. NIH 3T3 cells stably expressing the indicated mCherry-UnaG fusion proteins derived from error-prone PCR were treated with or without 20 μ M bilirubin (BR) for 24 h, and UnaG and mCherry fluorescence was monitored by analytical flow cytometry. WT = wild-type UnaG.

Figure 3.

Characterization of the FDD. (A) Fluorescence microscopy of NIH 3T3 cells stably expressing mCherry-FDD. Cells were treated with or without 10 μ M BR for 24 h then analyzed using epifluorescence microscopy. Scale bars, 20 μ m. (B) NIH 3T3 cells stably expressing the mCherry-FDD fusion were treated with the indicated concentrations of bilirubin (BR) for 18 h, and fluorescence was evaluated by flow cytometry. (C) NIH 3T3 cells stably expressing the mCherry-FDD fusion were treated with 10 μ M BR for 24 h, at which point cells were washed with media to remove the ligand. Decreases in fluorescence

were monitored by flow cytometry. (D) NIH 3T3 cells stably expressing mCherry-FDD were treated with 10 μ M BR for 24 h, at which point cells were washed with media to remove ligand and subsequently exposed to 10 μ M MG132 for 8 h, then analyzed by flow cytometry. (E) Lysates of cells described in D were prepared and immunoblotted with antibodies against hemagglutinin antigen (HA; the tag was inserted at the N-terminus of mCherry). α -Tubulin served as the loading control. MFI = mean fluorescence intensity.

Figure 4.

FDD generality. (A) The FDD was fused to either the C- or N-terminus of four different protein targets and independently transduced into NIH 3T3 cells. Cell populations were treated with or without 10 μ M BR for 24 h, after which lysates were prepared and immunoblotted with antibodies against HA (for zfand2a-FDD, the HA tag was inserted between zfand2a and FDD; for FDD-zfand2b and ube2n-FDD, the HA tag was appended to the N-terminus of each fusion protein) or against p21 (for FDD-p21). α -Tubulin served as the loading control. (B) NIH 3T3 cells stably expressing FDD-tagged zfand2a, zfand2b, and ube2n and transiently expressing p21 were treated with or without 10 μ M BR for 24 h then analyzed using epifluorescence microscopy. Scale bars, 20 μ m.